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(54) Title: ENDODERM, CARDIAC AND NEURAL INDUCING FACTORS (57) Abstract <p>Novel proteins have been designated "cerberus" and "frzb-1", respectively. Cerberus is expressed as a secreted peptide during embryogenesis of the <i>Xenopus</i> embryo, and is expressed specifically in the head organizer region. This new molecule has endodermal, cardiac, and neural tissue inducing activity, that should prove useful in therapeutic, diagnostic, and clinical applications requiring regeneration, differentiation, or repair of these and other tissues. Frzb-1 is a soluble antagonist of growth factors of the Wnt family that acts by binding to Wnt growth factors in the extracellular space. A third novel protein is termed PAPC which promotes the formation of dorsal mesoderm and somites in the embryo.</p>		

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**ENDODERM, CARDIAC AND
NEURAL INDUCING FACTORS**

5 **Field of the Invention**

 The invention generally relates to growth factors, neurotrophic factors, and their inhibitors, and more particularly to several new growth factors with neural, endodermal, and cardiac tissue inducing
10 activity, to complexes and compositions including the factors, and to DNA or RNA coding sequences for the factors. Further, one of the novel growth factors should be useful in tumor suppression gene therapy.

 This application claims the benefit of U.S.
15 Provisional Application No. 60/020,150, filed June 20, 1996.

 This invention was made with Government support under grant contract number HD-21502, awarded by the National Institutes of Health. The Government has
20 certain rights in this invention.

Background of the Invention

 Growth factors are substances, such as polypeptide hormones, which affect the growth of defined populations of animal cells *in vivo* or *in vitro*, but
25 which are not nutrient substances. Proteins involved in the growth and differentiation of tissues may promote or inhibit growth, and promote or inhibit differentiation, and thus the general term "growth factor" includes cytokines, trophic factors, and their inhibitors.

Widespread neuronal cell death accompanies normal development of the central and peripheral nervous systems. Studies of peripheral target tissues during development have shown that neuronal cell death results from the competition among neurons for limiting amounts of survivor factors ("neurotrophic factors"). The earliest identified of these, nerve growth factor ("NGF"), is the most fully characterized and has been shown to be essential for the survival of sympathetic and neural crest-derived sensory neurons during early development of both chick and rat.

One family of neurotropic factors are the Wnts, which have dorsal axis-inducing activity. Most of the Wnt proteins are bound to cell surfaces. (See, e.g., Sokol et al., *Science*, 249, pp. 561-564, 1990.) Dorsal axis-inducing activity in *Xenopus* embryos by one member of this family (Xwnt-8) was described by Smith and Harland in 1991, *Cell*, 67, pp. 753-765. The authors described using RNA injections as a strategy for identifying endogenous RNAs involved in dorsal patterning to rescue dorsal development in embryos that were ventralized by UV irradiation.

Another member of the growth and neurotropic factor family was subsequently discovered and described by Harland and Smith, which they termed "noggin." (*Cell*, 70, pp. 829-840 (1992).) Noggin is a good candidate to function as a signaling molecule in Nieuwkoop's center, by virtue of its maternal transcripts, and in Spemann's organizer, through its zygotic organizer-specific expression. Besides noggin, other secreted factors may be involved in the organizer phenomenon.

Another *Xenopus* gene designated "chordin" that begins to be expressed in Spemann's organizer and that can completely rescue axial development in ventralized

embryos was described by Sasai et al., *Cell*, 79, pp. 779-790, 1994. In addition to dorsalizing mesoderm, chordin has the ability to induce neural tissue and its activities are antagonized by Bone Morphogenetic Protein-4 (Sasai et al., *Nature*, 376, pp. 333-336, 1995).

Therefore, the dorsal lip or Spemann's organizer of the *Xenopus* embryo is an ideal tissue for seeking novel growth and neurotrophic factors. New growth and neurotrophic factors are useful agents, particularly those that are secreted due to their ability to be used in physiologically active, soluble forms because these factors, their receptors, and DNA or RNA coding sequences therefore and fragments thereof are useful in a number of therapeutic, clinical, research, diagnostic, and drug design applications.

Summary of the Invention

In one aspect of the present invention, the sequence of the novel peptide that can be in substantially purified form is shown by SEQ ID NO:1. The *Xenopus* derived SEQ ID NO:1 has been designated "cerberus," and this peptide is capable of inducing endodermal, cardiac, and neural tissue development in vertebrates when expressed. The nucleotide sequence which, when expressed results in cerberus, is illustrated by SEQ ID NO:2. Since peptides of the invention induce endodermal, cardiac, and neural tissue differentiation in vertebrates, they should be able to be prepared in physiologically active form for a number of therapeutic, clinical, and diagnostic applications.

Cerberus was isolated during a search for molecules expressed specifically in Spemann's organizer containing a secretory signal sequence. In addition to cerberus, two other novel cDNAs were identified.

The *Xenopus* derived peptide that can be deduced from SEQ ID NO:3 encodes a novel protein we had earlier designated as "frazzled," a secreted protein of 318 amino acids that has dorsalizing activity in *Xenopus* embryos. We now designate the novel protein as "frzb-1." The gene for frzb-1 is expressed in many adult tissues of many animals, three of the cDNAs (*Xenopus*, mouse, and human) have been cloned by us. The accession numbers for the *Xenopus*, mouse, and human frzb-1 cDNA sequences of the gene now designated frzb-1 are U68059, U68058, and U68057, respectively. Frzb-1 has some degree of sequence similarity to the *Drosophila* gene frizzled which has been shown to encode a seven-transmembrane protein that can act both as a signalling and as a receptor protein (Vinson et al., *Nature*, 338, pp. 263-264, 1989; Vinson and Adler, *Nature*, 329, pp. 549-551, 1987). Vertebrate homologues of Frizzled have been isolated and they too were found to be anchored to the cell membrane by seven membrane spanning domains (Wang et al., *J. Biol. Chem.*, 271, pp. 4468-4476, 1996). Frzb-1 differs from the frizzled proteins in that it is an entirely soluble, diffusible secreted protein and therefore suitable as a therapeutic agent. The nucleotide sequence derived from *Xenopus* that, when expressed, results in frzb-1 protein is illustrated by SEQ ID NO:4. The frzb-1 protein derived from mouse is shown as SEQ ID NO:7, while the mouse frzb-1 nucleotide sequence is SEQ ID NO:8. The human derived frzb-1 protein is illustrated by SEQ ID NO:9, and the human frzb-1 nucleotide sequence is SEQ ID NO:10.

Frzb-1 is an antagonist of Wnts *in vivo*, and thus is believed to find utility as a tumor suppressor gene, since overexpressed Wnt proteins cause cancer. Frzb-1 may also be a useful vehicle for solubilization

and therapeutic delivery of Wnt proteins complexed with it.

The final cDNA isolated containing a signal sequence results in a peptide designated Paraxial Protocadherin (PAPC). The cDNA for PAPC is a divergent member of the cadherin multigene family. PAPC is most related to protocadherin 43 reported by Sano et al., *The EMBO J.*, 12, pp. 2249-2256, 1993. As shown in SEQ ID NO:5, the PAPC gene encodes a transmembrane protein of 896 amino acids, of which 187 are part of an intracellular domain. PAPC is a cell adhesion molecule, and microinjection of PAPC mRNA constructs into *Xenopus* embryos suggest that PAPC acts as a molecule involved in mesoderm differentiation. A soluble form of the PAPC extracellular domain is able to block muscle and mesoderm formation in *Xenopus* embryos. The nucleotide sequence encoding *Xenopus* PAPC is provided in SEQ ID NO:6.

Cerberus, frzb-1, or PAPC or fragments thereof (which also may be synthesized by *in vitro* methods) may be fused (by recombinant expression or *in vitro* covalent methods) to an immunogenic polypeptide and this, in turn, may be used to immunize an animal in order to raise antibodies against the novel proteins. Antibodies are recoverable from the serum of immunized animals. Alternatively, monoclonal antibodies may be prepared from cells from the immunized animal in conventional fashion. Immobilized antibodies are useful particularly in the diagnosis (*in vitro* or *in vivo*) or purification of cerberus, frzb-1, or PAPC.

Substitutional, deletional, or insertional mutants of the novel polypeptides may be prepared by *in vitro* or recombinant methods and screened for immuno-crossreactivity with cerberus, frzb-1, or PAPC and for cerberus antagonist or agonist activity.

Cerberus or frzb-1 also may be derivatized in vitro in order to prepare immobilized and labelled proteins, particularly for purposes of diagnosis of insufficiencies thereof, or for affinity purification of antibodies thereto.

Among applications for the novel proteins are tissue replacement therapy and, because frzb-1 is an antagonist of Wnt signaling, tumor suppression therapies. The cerberus receptor may define a novel signalling pathway. In addition, frzb-1 could permit the isolation of novel members of the Wnt family of growth factors.

Brief Description of the Drawings

Figure 1 illustrates the amino acid sequence (SEQ ID NO:1) of the Fig. 2 cDNA clone for cerberus;

Figure 2 illustrates a cDNA clone (SEQ ID NO:2) for cerberus derived from Xenopus. Sense strand is on top (5' to 3' direction) and the antisense strand on the bottom line (in the opposite direction);

Figures 3 and 4 show the amino acid and nucleotide sequence, respectively, of full-length frzb-1 from Xenopus (SEQ ID NOS:3 and 4);

Figures 5 and 6 show the amino acid and nucleotide sequence, respectively, of full-length PAPC from Xenopus (SEQ ID NOS:5 and 6);

Figures 7 and 8 show the amino acid and nucleotide sequence, respectively, of full-length frzb-1 from mouse (SEQ ID NOS:7 and 8); and

Figures 9 and 10 show the amino acid and nucleotide sequence, respectively, of full-length frzb-1 from human (SEQ ID NOS:9 and 10).

Detailed Description of the Preferred Embodiments

Among the several novel proteins and their nucleotide sequences described herein, is a novel endodermal, cardiac, and neural inducing factor in vertebrates that we have named "cerberus." When referring to cerberus, the present invention also contemplates the use of fragments, derivatives, agonists, or antagonists of cerberus molecules. Because cerberus has no homology to any reported growth factors, it is proposed to be the founding member of a novel family of growth factors with potent biological activities, which may be isolated using SEQ ID NO:2.

The amphibian organizer consists of several cell populations with region-specific inducing activities. On the basis of morphogenetic movements, three very different cell populations can be distinguished in the organizer. First, cells with crawling migration movements involute, fanning out to form the prechordal plate. Second, cells involute through the dorsal lip driven by convergence and extension movements, giving rise to the notochord of the trunk. Third, involution ceases and the continuation of mediolateral intercalation movements leads to posterior extension movements and to the formation of the tail notochord and of the chordoneural hinge. The three cell populations correspond to the head, trunk, and tail organizers, respectively.

The cerberus gene is expressed at the right time and place to participate in cell signalling by Spemann's organizer. Specifically, cerberus is expressed in the head organizing region that consists of crawling-migrating cells. The cerberus expressing region corresponds to the prospective foregut, including the liver and pancreas anlage, and the heart mesoderm.

Cerberus expression is activated by chordin, noggin, and organizer-specific homeobox genes.

Our studies were conducted in early embryos of the frog *Xenopus laevis*. The frog embryo is well suited to experiments, particularly experiments pertaining to generating and maintaining regional differences within the embryo for determining roles in tissue differentiation. It is easy to culture embryos with access to the embryos even at very early stages of development (preceding and during the formation of body pattern and differentiation) and the embryos are large. The initial work with noggin and chordin also had been in *Xenopus* embryos, and, as predicted, was highly conserved among vertebrates. Predictions based on work with *Xenopus* as to corresponding human noggin were proven true and the ability to clone the gene for human noggin was readily accomplished. (See the description of *Xenopus* work and cloning information in PCT application, published March 17, 1994, WO 9 405 800, and the subsequent human cloning based thereon in the PCT application, also published March 17, 1994, as WO 9 405 791.)

CLONING

The cloning of cerberus, frzb-1, and PAPC resulted from a comprehensive screen for cDNAs enriched in Spemann's organizer. Subtractive differential screening was performed as follows. In brief, poly A⁺ RNA was isolated from 300 dorsal lip and ventral marginal zone (VMZ) explants at stage 10½. After first strand cDNA synthesis approximately 70-80% of common sequences were removed by subtraction with biotinylated VMZ poly A⁺ RNA prepared from 1500 ventral gastrula halves. For differential screening, duplicate filters (2000 plaques per 15 cm plate, a total of 80,000 clones

screened) of an unamplified oriented dorsal lip library were hybridized with radiolabeled dorsal lip or VMZ cDNA. Putative organizer-specific clones were isolated, grouped by sequence analysis from the 5' end and whole-mount in situ hybridization, and subsequently classified into known and new dorsal-specific genes. Rescreening of the library (100,000 independent phages) with a cerberus probe resulted in the isolation of 45 additional clones, 31 of which had similar size as the longest one of the 11 original clones indicating that they were presumably full-length cDNAs. The longest cDNAs for cerberus, frzb-1, and PAPC were completely sequenced.

To explore the molecular complexity of Spemann's organizer we performed a comprehensive differential screen for dorsal-specific cDNAs. The method was designed to identify abundant cDNAs without bias as to their function. As shown in Table 1, five previously known cDNAs and five new ones were isolated, of which three (expressed as cerberus, frzb-1, and PAPC, respectively) had secretory signal sequences.

TABLE 1

	Previously Known Genes	Gene Product	No. of Isolates
	Chordin	novel secreted protein	70
	Goosecoid	homeobox gene	3
5	Pintallavis/XFKH-1	forkhead/transcription factor	2
	Xnot-2	homeobox gene	1
	Xlim-1	homeobox gene	1
	New Genes		
	Cerberus	novel secreted protein	11
10	PAPC	cadherin-like/transmembrane	2
	Frzb-1	novel secreted protein	1
	Sox-2	sry/transcription factor	1
	Fkh-like	forkhead/transcription factor	1

15 The most abundant dorsal-specific cDNA was chordin (chd), with 70 independent isolates. The second most abundant cDNA was isolated 11 times and named cerberus (after a mythological guardian dog with multiple heads). The cerberus cDNA encodes a putative secreted polypeptide of 270 amino acids, with an amino

20 terminal hydrophobic signal sequence and a carboxy terminal cysteine-rich region (Fig. 1). Cerberus is expressed specifically in the head organizer region of the *Xenopus* embryo, including the future foregut.

25 An abundant mRNA found in the dorsal region of the *Xenopus* gastrula encodes the novel putative secreted protein we have designated as cerberus. Cerberus mRNA has potent inducing activity in *Xenopus* embryos, leading to the formation of ectopic heads. Unlike other organizer-specific factors, cerberus does not dorsalize

30 mesoderm and is instead an inhibitor of trunk-tail mesoderm. Cerberus is expressed in the anterior-most

domain of the gastrula including the leading edge of the deep layer of the dorsal lip a region that, as shown here, gives rise to foregut and midgut endoderm. Cerberus promotes the formation of cement gland, olfactory placodes, cyclopic eyes, forebrain, and duplicated heart and liver (a foregut derivative). Because the pancreas is also derived from this foregut region, it is likely that cerberus induces pancreas in addition to liver. The expression pattern and inducing activities of cerberus suggest a role for a previously neglected region of the embryo, the prospective foregut endoderm, in the induction of the anterior head region of the embryo.

Turning to Fig. 1, *Xenopus cerberus* encodes a putative secreted protein transiently expressed during embryogenesis and the deduced amino acid sequence of *Xenopus cerberus* is shown. The signal peptide sequence and the nine cysteine residues in the carboxy-terminus are indicated in bold. Potential N-linked glycosylation sites are underlined. In database searches the cerberus protein showed limited similarity only to the mammalian Dan protein, a possible tumor suppressor proposed to be a DNA-binding protein.

Cerberus appears to be a pioneer protein, as its amino acid sequence and the spacing of its 9 cysteine residues were not significantly similar to other proteins in the databases (NCBI-Gen Bank release 93.0). We conclude that the second most abundant dorsal-specific cDNA encodes a novel putative secreted factor, which should be the founding member of a novel family of growth factors active in cell differentiation.

Cerberus Demarcates an Anterior Organizer Domain. Cerberus mRNA is expressed at low levels in the unfertilized egg, and zygotic transcripts start accumulating at early gastrula. Expression continues

during gastrula and early neurula, rapidly declining during neurulation. Importantly, cerberus expression starts about one hour after that of chd, suggesting that cerberus could act downstream of the chd signal.

5 Whole-mount *in situ* hybridizations reveal that expression starts in the yolky endomesodermal cells located in the deep layer of the organizer. The cerberus domain includes the leading edge of the most anterior organizer cells and extends into the lateral
10 mesoderm. The leading edge gives rise to liver, pancreas, and foregut in its midline, and the more lateral region gives rise to heart mesoderm at later stages of development.

Fig. 2 sets out the sequence of a full length
15 *Xenopus* cDNA for cerberus.

This entirely new molecule has demonstrated physiological properties that should prove useful in therapeutic, diagnostic, and clinical applications that require regeneration, differentiation, or repair of
20 tissues, such wound repair, neuronal regeneration or transplantation, supplementation of heart muscle differentiation, differentiation of pancreas and liver, and other applications in which cell differentiation processes are to be induced.

25 The second, novel, secreted protein we have discovered is called "frzb-1," which was shown to be a secreted protein in *Xenopus* oocyte microinjection experiments. Thus it provides a natural soluble form of the related extracellular domains of *Drosophila* and
30 vertebrate frizzled proteins. We propose that the latter proteins could be converted into active soluble forms by introducing a stop codon before the first transmembrane domain. We have noted that the cysteine-rich region of frzb-1 and frizzled contains some overall
35 structural homology with Wnt proteins using the Profile

Search homology program (Gribskov, *Meth. Enzymol.*, 183, pp. 146-159, 1990). This had raised the interesting possibility that frzb-1 could interact directly with Wnt growth factors in the extracellular space. This was
5 because we had found that when microinjected into xenopus embryos, frzb-1 constructs have moderate dorsalizing activity, leading to the formation of embryos with enlarged brain and head, and shortened trunk. Somatic muscle differentiation, which requires
10 Xwnt-8, was inhibited. In the case of frzb-1, an attractive hypothesis, suggested by the structural homologies, was that it may act as an inhibitor of Wnt-8, a growth factor that has ventralizing activity in the Xenopus embryo (Christian and Moon, *Genes Dev.*, 7,
15 pp. 13-28, 1993). We have shown that frzb-1 can interact with Xwnt-8 and Wnt-1, and it is expected that it could also interact with other members of the Wnt family of growth factors, of which at least 15 members exist in mammals. In addition, a possible interaction
20 with Wnts was suggested by the recent discovery that dishevelled, a gene acting downstream of wingless, has strong genetic interaction with frizzled mutants in *Drosophila* (Krasnow et al., *Development*, 121, pp. 4095-4102, 1995). This possibility has been explored in
25 depth (Leyns et al., *Cell*, 88, pp. 747-756, March 21, 1997), because a soluble antagonist of the Wnt family of proteins is expected to be of great therapeutic value. Examples 1 and 2 illustrate tests that show antagonism of Xwnt-8 by binding to frzb-1.

30 Vertebrate homologues of Frizzled have been isolated and they too are anchored to the cell membrane by seven membrane spanning domains (Wang et al., *J. Biol. Chem.*, 271, pp. 4468-4476, 1996). Frzb-1 differs from the frizzled proteins in that it is an
35 entirely soluble, diffusible secreted protein and

therefore suitable as a therapeutic agent. The nucleotide sequence that when expressed results in frzb-1 protein is illustrated by SEQ ID NO:4.

SEQ ID NO:4 corresponds to the *Xenopus* homolog, but by using it in BLAST searches (and by cloning mouse frzb-1) we had been able to assemble the sequence of the entire mature human frzb-1 protein, SEQ ID NO:9. Indeed, human frzb-1 is encoded in six expressed sequence tags (ESTs) available in Genbank. The human frzb-1 sequence can be assembled by overlapping in the 5' to 3' direction the ESTs with the following accession numbers in Genbank: H18848, R63748, W38677, W44760, H38379, and N71244. No function had yet been assigned to these EST sequences, but we believe and thus propose here that human frzb-1 will have similar functions in cell differentiation to those described above for *Xenopus* frzb-1. The nucleotide sequence of human frzb-1 is shown in SEQ ID NO:10. The mouse frzb-1 protein and nucleotide sequences are provided by SEQ ID NOS:7 and 8, respectively.

In particular, we believe that frzb-1 will prove useful in gene therapy of human cancer cells. In this rapidly developing field, one approach is to introduce vectors expressing anti-sense sequences to block expression of dominant oncogenes and growth factor receptors. Another approach is to produce episomal vectors that will replicate in human cells in a controlled fashion without transforming the cells. For an example of the latter (an episomal expression vector system for human gene therapy), reference is made to U.S. Patent 5,624,820, issued April 29, 1997, inventor Cooper.

Gene therapy now includes uses of human tumor suppression genes. For example, U.S. Patent 5,491,064, issued February 13, 1996, discloses a tumor suppression

gene localized on chromosome 11 and described as potentially useful for gene therapy in cancers deleted or altered in their expression of that gene. Frzb-1 maps to chromosome 2q31-33 and loss of one copy of the 2q31-33 and loss of one copy of the 2q arm has been observed with high incidence in lung carcinomas, colo-rectal carcinomas, and neuroblastomas, which has lead to the proposal that the 2q arm carries a tumor suppressor gene. We expect frzb to be a tumor suppressor gene, and thus to be useful in tumor suppression applications.

A number of applications for cerberus and frzb-1 are suggested from their pharmacological (biological activity) properties.

For example, the cerberus and frzb-1 cDNAs should be useful as a diagnostic tool (such as through use of antibodies in assays for proteins in cell lines or use of oligonucleotides as primers in a PCR test to amplify those with sequence similarities to the oligonucleotide primer, and to determine how much of the novel protein is present).

Cerberus, of course, might act upon its target cells via its own receptor. Cerberus, therefore, provides the key to isolate this receptor. Since many receptors mutate to cellular oncogenes, the cerberus receptor should prove useful as a diagnostic probe for certain tumor types. Thus, when one views cerberus as ligand in complexes, then complexes in accordance with the invention include antibody bound to cerberus, antibody bound to peptides derived from cerberus, cerberus bound to its receptor, or peptides derived from cerberus bound to its receptor or other factors. Mutant forms of cerberus, which are either more potent agonists or antagonists, are believed to be clinically useful.

Such complexes of cerberus and its binding protein partners will find uses in a number of applications.

Practice of this invention includes use of an oligonucleotide construct comprising a sequence coding
5 for cerberus or frzb-1 and for a promoter sequence
operatively linked in a mammalian or a viral expression
vector. Expression and cloning vectors contain a
nucleotide sequence that enables the vector to replicate
in one or more selected host cells. Generally, in
10 cloning vectors this sequence is one that enables the
vector to replicate independently of the host
chromosomes, and includes origins of replication or
autonomously replicating sequences. The well-known
plasmid pBR322 is suitable for most gram negative
15 bacteria, the 2 μ plasmid origin for yeast and various
viral origins (SV40, polyoma, adenovirus, VSV or BPV)
are useful for cloning vectors in mammalian cells.

Expression and cloning vectors should contain
a selection gene, also termed a selectable marker.
20 Typically, this is a gene that encodes a protein
necessary for the survival or growth of a host cell
transformed with the vector. The presence of this gene
ensures that any host cell which deletes the vector will
not obtain an advantage in growth or reproduction over
25 transformed hosts. Typical selection genes encode
proteins that (a) confer resistance to antibiotics or
other toxins, e.g. ampicillin, neomycin, methotrexate or
tetracycline, (b) complement auxotrophic deficiencies.

Examples of suitable selectable markers for
30 mammalian cells are dihydrofolate reductase (DHFR) or
thymidine kinase. Such markers enable the identifica-
tion of cells which were competent to take up the
cerberus nucleic acid. The mammalian cell transformants
are placed under selection pressure which only the
35 transformants are uniquely adapted to survive by virtue

of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of cerberus or frzb-1 can therefor be synthesized from the amplified DNA.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants in a culture medium which contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell in this case is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub and Chasin, *Proc. Nat. Acad. Sci.*, 77, 4216 (1980). The transformed cells then are exposed to increased levels of Mtx. This leads to the synthesis of multiple copies of the DHFR gene and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding cerberus or frzb-1. Alternatively, host cells transformed by an expression vector comprising DNA sequences encoding cerberus or frzb-1 and aminoglycoside 3' phosphotransferase (APH) protein can be selected by cell growth in medium containing an aminoglycosidic antibiotic such as kanamycin or neomycin or G418. Because eukaryotic cells do not normally express an endogenous APH activity, genes encoding APH protein, commonly referred to as neo resistant genes, may be used as dominant selectable markers in a wide range of eukaryotic host cells, by which cells transformed by the vector can readily be identified.

Expression vectors, unlike cloning vectors, should contain a promoter which is recognized by the host organism and is operably linked to the cerberus nucleic acid. Promoters are untranslated sequences located upstream from the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of nucleic acid under their control. They typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g. the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters can be operably linked to cerberus encoding DNA by removing them from their gene of origin by restriction enzyme digestion, followed by insertion 5' to the start codon for cerberus or frzb-1.

Nucleic acid is operably linked when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein which participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, operably linked means that the DNA sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not

exit then synthetic oligonucleotide adapters or linkers are used in accord with conventional practice.

Transcription of the protein-encoding DNA in mammalian host cells is controlled by promoters obtained
5 from the genomes of viruses such as polyoma, cytomegalovirus, adenovirus, retroviruses, hepatitis-B virus, and most preferably Simian Virus 40 (SV40), or from heterologous mammalian promoters, e.g. the actin promoter. Of course, promoters from the host cell or
10 related species also are useful herein.

Cerberus and frzb-1 are clearly useful as a component of culture media for use in culturing cells, such as endodermal, cardiac, and nerve cells, *in vitro*. We believe cerberus and frzb-1 will find uses as agents
15 for enhancing the survival or inducing the growth of liver, pancreas, heart, and nerve cells, such as in tissue replacement therapy.

The final cDNA isolated containing a signal sequence results in a peptide designated Paraxial
20 Protocadherin (PAPC). The cDNA for PAPC is a divergent member of the cadherin multigene family. PAPC is most related to protocadherin 43 reported by Sano et al., *The EMBO J.*, 12, pp. 2249-2256, 1993. As shown in SEQ ID NO:5, the PAPC gene encodes a transmembrane protein of
25 896 amino acids, of which 187 are part of an intracellular domain. PAPC is a cell adhesion molecule, and microinjection of PAPC mRNA constructs into *Xenopus* embryos suggest that PAPC acts in mesoderm differentiation. The nucleotide sequence encoding
30 *Xenopus* PAPC is provided in SEQ ID NO:6.

Therapeutic formulations of the novel proteins may be prepared for storage by mixing the polypeptides having the desired degree of purity with optional physiologically acceptable carriers, excipients or
35 stabilizers, in the form of lyophilized cake or aqueous

solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; anti-oxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins. Other components can include glycine, glutamine, asparagine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronic or PEG.

Polyclonal antibodies to the novel proteins generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of cerberus or frzb-1 and an adjuvant. It may be useful to conjugate these proteins or a fragment containing the target amino acid sequence to a protein which is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl_2 , or $\text{R}'\text{N} = \text{C} = \text{NR}$.

Animals can be immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1 μg of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally in multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freund's complete

adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later animals are bled and the serum is assayed for anti-cerberus titer. Animals are boosted until the titer plateaus. Preferably, the animal is
5 boosted with the conjugate of the same cerberus or frzb-1 polypeptide, but conjugated to a different protein and/or through a different cross-linking agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as
10 alum are used to enhance the immune response.

Monoclonal antibodies are prepared by recovering spleen cells from immunized animals and immortalizing the cells in conventional fashion, e.g. by fusion with myeloma cells or by EB virus transformation
15 and screening for clones expressing the desired antibody.

Antibodies are useful in diagnostic assays for cerberus, frzb-1, or PAPC or their antibodies and to identify family members. In one embodiment of a
20 receptor binding assay, an antibody composition which binds to all of a selected plurality of members of the cerberus family is immobilized on an insoluble matrix, the test sample is contacted with the immobilized antibody composition in order to adsorb all cerberus
25 family members, and then the immobilized family members are contacted with a plurality of antibodies specific for each member, each of the antibodies being individually identifiable as specific for a predetermined family member, as by unique labels such as
30 discrete fluorophores or the like. By determining the presence and/or amount of each unique label, the relative proportion and amount of each family member can be determined.

The antibodies also are useful for the
35 affinity purification of the novel proteins from

recombinant cell culture or natural sources. Antibodies that do not detectably cross-react with other growth factors can be used to purify the proteins free from these other family members.

5

EXAMPLE 1

Frzb-1 Antagonizes Xwnt-8 Non-Cell Autonomously

To test whether frzb-1 can antagonize secondary axes caused by Xwnt-8 after secretion by injected cells, an experimental design was used. Thus, frzb-1 mRNA was injected into each of the four animal blastomeres of eight-cell embryos, and subsequently, a single injection of Xwnt-8 mRNA was given to a vegetal-ventral blastomere at the 16-32 cell stage. In two independent experiments, we found that injection of frzb-1 alone (n=13) caused mild dorsalization with enlargement of the cement gland in all embryos and that injection of Xwnt-8 alone (n=53) lead to induction of complete secondary axes in 67% of the embryos. However, injection of frzb-1 into animal caps abolished the formation of complete axes induced by Xwnt-8 (n=27), leaving only a residual 14% of embryos with very weak secondary axes. The double-injected embryos retained the enlarged cement gland phenotype caused by injection of frzb-1 mRNA alone. Because both mRNAs encode secreted proteins and were microinjected into different cells, we conclude that the antagonistic effects of frzb-1 and Xwnt-8 took place in the extracellular space after these proteins were secreted.

EXAMPLE 2

Membrane-Anchored Wnt-1 Confers Frzb-1 Binding

To investigate a possible interaction between frzb-1 and Wnts, the first step was to insert an HA epitope tag into a Xenopus frzb-1 construct driven by the CMV (cytomegalovirus) promoter. Frzbl-HA was tested in mRNA microinjection assays in Xenopus embryos and found to be biologically active. Conditioned medium from transiently transfected cells contained up to 10 μ g/ml of Frzbl-HA (quantitated on Western blots using an HA-tagged protein standard).

Transient transfection of 293 cells has been instrumental in demonstrating interactions between wingless and frizzled proteins. We therefore took advantage of constructs in which Wnt-1 was fused at the amino terminus of CD8, generating a transmembrane protein containing biologically active Wnt-1 exposed to the extracellular compartment. A Wnt1CD8 cDNA construct (a generous gift of Dr. H. Varmus, NIH) was subcloned into the pCDNA (Invitrogen) vector and transfected into 293 cells. After incubation with Frzbl-HA-conditioned medium (overnight at 37°C), intensely labeled cells were observed by immunofluorescence. As a negative control, a construct containing 120 amino acids of Xenopus chordin, an unrelated secreted protein was used. Transfection of this construct produced background binding of Frzbl-HA to the extracellular matrix, both uniform and punctate. Cotransfection of Wnt1CD8 with pCDNA-LacZ showed that transfected cells stained positively for Frzbl-HA and LacZ. Since Wnt1CD8 contains the entire CD8 molecule, a CD8 cDNA was used as an additional negative control. After transfection with LacZ and full-length CE8, Frzbl-HA failed to bind to the transfected cells. Although most of our experiments

were carried out at 37°C, Frzb1-HA-conditioned medium also stained Wnt1CD8-transfected cells after incubation at 4°C for 2 hours.

Attempts to biochemically quantitate the binding of Frzb-1 to Wnt1CD8-transfected cells were unsuccessful due to high background binding to control cultures, presumably due to binding to the extracellular matrix. Thus, we were unable to estimate a K_D for the affinity of the Frzb-1/Wnt-1 interaction. However, when serial dilutions of conditioned medium containing Frzb1-HA were performed (ranging from 2.5×10^{-7} to 1.25×10^{-10} M), staining of Wnt1CD8-transfected cells was found at all concentrations.

Although we have been unable to provide biochemical evidence for direct binding between Wnts and frzb-1, this cell biological assay indicates that Frzb1-HA can bind, directly or indirectly, to Wnt-1 on the cell membrane in the 10^{-10} M range.

It is to be understood that while the invention has been described above in conjunction with preferred specific embodiments, the description and examples are intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.

It is Claimed:

1. A substantially pure protein characterized by a physiologically active form and comprising an amino acid sequence encoded by the DNA of SEQ ID NO:2.
2. The protein as in claim 1 having neurotrophic, growth or differentiation factor activity.
3. A composition comprising the protein of claim 1 and a physiologically acceptable carrier with which the peptide is admixed.
4. An oligonucleotide construct comprising a sequence coding for a protein and an expression vector operatively linked therewith, the protein having neurotrophic, growth or differentiation factor activity
5 and being expressible from SEQ ID NO:2.
5. The construct as in claim 4 wherein the expression vector is a mammalian or viral expression vector.
6. A substantially pure protein characterized by a physiologically active form and comprising an amino acid sequence encoded by the DNA of SEQ ID NO:4, SEQ ID NO:8, or SEQ ID NO:10.
7. The protein as in claim 6 having neurotrophic, growth or differentiation factor activity.
8. A composition comprising the protein of claim 6 and a physiologically acceptable carrier with which the protein is admixed.

9. An oligonucleotide construct comprising a sequence coding for a protein and an expression vector operatively linked therewith, the protein being expressible from SEQ ID NO:4, SEQ ID NO:8 or SEQ ID NO:10.

5

10. The construct as in claim 9 wherein the protein is expressible in soluble form.

11. The construct as in claim 9 wherein the expression vector is a mammalian or viral expression vector.

12. A complex comprising a substantially pure frzb-1 protein complexed with at least one Wnt protein.

13. A substantially pure protein characterized by a physiologically active form and comprising an amino acid sequence encoded by the DNA of SEQ ID NO:6.

14. The protein as in claim 13 having mesoderm differentiation activity.

15. A composition comprising the protein of claim 13 and a physiologically acceptable carrier with which the protein is admixed.

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MLNLVLRICI	IVCLVNDGAG	KHSEGRERTK	TYSLNSRGYF	40
RKERGARRSK	ILLVNTKGLD	EPHIGHGDFG	LVAELFDSTR	80
THTNRKEPDM	NKVKLFSTVA	HGN <u>K</u> SARRKA	YNGSRRNIFS	120
RRSFDKRNTE	VTEKPGAKMF	WNNFLVKMNG	APONT <u>S</u> HGSK	160
AQEIMKEACK	TLPFTQNIVH	ENCDRMVION	NLCFGKCISL	200
HVPNQODRRN	TCSHCLPSKF	TLNHLTL <u>NCT</u>	GSKNVVKVVM	240
MVEECTCEAH	KSNFHQTAQF	NMDTSTTLHH		270

Figure 1

SUBSTITUTE SHEET (RULE 26)

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GAATTCCCAG CAAGTCGCTC AGAAACACTG CAGGGTCTAG ATATCATACA ATGTTACTAA	60
CTTAAGGGTC GTTCAGCGAG TCTTTGTGAC GTCCCAGATC TATAGTATGT TACAATGATT	
ATGTAICTAG GATCTGTATT ATCGTCTGCC TTGTGAATGA TGGAGCAGGA AAACACTCAG	120
TACATGAGTC CTAGACATAA TAGCAGACGG AACACTTACT ACCTCGTCCT TTTGTGAGTC	
AAGGACGAGA AAGGACAAAA ACATATTTCAC TTAACAGCAG AGGTTACTTC AGAAAAGAAA	180
TTCTGCTCT TTTCTGTTTT TGTATAAGTG AATTGTCGTC TCCAATGAAG TCTTTTCTTT	
GAGGAGCAGC TAGGAGCAAG ATTCTGCTGG TGAATACTAA AGGTCTTGAT GAACCCACAC	240
CTCTCGTGC ATCCTCGTTC TAAGACGACC ACTTATGATT TCCAGAACTA CTGGGGGTGT	
TTGGGCATGG TGATTTTCGC TTAGTAGCTG AACTATTTGA TTCCACCAGA ACACATACAA	300
AACCGTACC ACTAAAAGCG AATCATCGAC TTGATAAACT AAGGTGGTCT TGTGTATGTT	
ACAGAAAAGA GCCAGACATG AACAAAGTCA AGCTTTTCTC AACAGTTGCC CATGGAAACA	360
TGCTTTTCT CGGTCTGTAC TTGTTTCAGT TCGAAAAGAG TTGTCAACGG GTACCTTTGT	
AAAGTGCAAG AAGAAAAGCT TACAATGGTT CTAGAAGGAA TATTTTTOCT CGCGTTCTTT	420
TTTCACGTTT TTCTTTTGA ATGTTACCAA GATCTTCCTT ATAAAAAGGA GCGGCAAGAA	
TTGATAAAG AAATACAGAG GTTACTGAAA AGCCTGGTGC CAAGATGTTT TGGAAACAATT	480
AACTATTTTC TTTATGTCTC CAATGACTTT TCGGACCACG GTTCTACAAG ACCTTGTTAA	
TTTTGGTTAA AATGAATGGA GCCCCACAGA ATACAAGCCA TGGCAGTAAA GCACAGGAAA	540
AAAACCAATT TTAATTACCT CGGGGTGTCT TATGTTGGT ACCGTCAATT CGTGTCTTTT	
TAATGAAAGA AGCTTGCAAA ACCTTGTTTT TCACTCAGAA TATTGTACAT GAAAACGTG	600
ATTACTTTCT TCGAACGTTT TGGAAACAAA AGTGAGTCTT ATAACATGTA CTTTGTACAC	
ACAGGATGGT GATACAGAAC AATCTGTGCT TTGGTAAATG CATCTCTCTC CATGTTCCAA	660
TGTCCTACCA CTATGTCTTG TTAGACAAGA AACCATTTAC GTAGAGAGAG GTACAAGGTT	
ATCAGCAAGA TCGACGAAAT ACTTGTTCCC ATTGCTTGCC GTCCAAATTT ACCCTGAACC	720
TAGTCGTTCT AGCTGCTTTA TGAACAAGGG TAACGAACGG CAGGTTTAAA TGGGACTTGG	
ACCTGACGCT GAATTGTACT GGATCTAAGA ATGTAGTAAA GGTGTGTCATG ATGGTAGAGG	780
TGGACTGCGA CTTAACATGA CCTAGATTCT TACATCATT CCAACAGTAC TAACATCTCC	
AATGCAAGTG TGAAGCTCAT AAGAGCAACT TCCACCAAC TGCACAGTTT AACATGGATA	840
TTAAGTGCAC ACTTCGAGTA TTCTGTTGA AGGTGGTTTG ACGTGTCAAA TTGTACCTAT	
CATCTACTAC CCTGCACCAT TAAAGGACTG CCATACAGTA TGGAAATGCC CTTTGTGTGG	900
GTAGATGATG GGACGTGGTA ATTTCTGAC GGTATGTCAT ACCTTTACGG GAAAACAACC	
AATATTTGTT ACATACTATG CATCTAAAGC ATTATGTTGC CTTCTATTTT ATATAACCA	960
TTATAAACAA TGTATGATAC GTAGATTTTC TAATACAACG GAAGATAAAG TATATTGGTG	
ATGGAATAAG GATTGTATGA ATTATAATTA ACAAATGGCA TTTTGTGTAA CATGCAAGAT	1020
TACCTTATTC CTAACATACT TAATATTAAAT TGTTTACCGT AAAACACATT GTACGTTCTA	

Figure 2A

SUBSTITUTE SHEET (RULE 28)

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CTCTGTTCCA TCAGTTGCAA GATAAAAGGC AATATTTGTT TGACTTTTTT TCTACAAAT 1080
GAGACAAGGT AGTCAACGTT CTATTTTCCG TTATAACAA ACTGAAAAA AGATGTTTTA

GAATACCCAA ATATATGATA AGATAATGGG GTCAAACTG TTAAGGGGTA ATGTAATAAT 1140
CTTATGGGTT TATATACTAT TCTATTACCC CAGTTTTGAC AATTCCCAT TACATTATTA

AGGGACTAAG TTTGCCCAGG AGCAGTGACC CATAACAACC AATCAGCAGG TATGATTTAC 1200
TCCTGATTC AAACGGGTCC TCGTCACTGG GTATTGTTGG TTAGTCGTCC ATACTAAATG

TGGTCACCTG TTTAAAAGCA AACATCTTAT TGGTTGCTAT GGGTTACTGC TTCTGGGCRA 1260
ACCAGTGGAC AAATTTTCGT TTGTAGAATA ACCAACGATA CCCAATGACG AAGACCCGTT

AATGTGTGCC TCATAGGGGG GTTAGTGTGT TGTGTACTGA ATAAATTGTA TTTATTTTAT 1320
TTACACACGG AGTATCCCC CAATCACACA ACACATGACT TATTTAACAT AAATAAAGTA

TGTTACAAAA AAAAAAAA
ACAAATGTTTT TTTTTTTT

Figure 2B

SUBSTITUTE SHEET (RULE 26)

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MSRTRKVDL LLLAIPGLAL LLLPNAYCAS CEPVRIPMCK SMPWNMTKMP NHLHHSTQAN	60
AILAIEQFEG LLTTECSQDL LFFLCAMYAP ICTIDFQHEP IKPCKSV CER ARAGCEPILI	120
KYRHTWPESL ACEELPVYDR GVCISPEAIV TVEQGTDSMP DFSMDSNNGN CGSGREHCKC	180
KPMKATQKTY LKNNYNYVIR AKVKEVKVVC HDATAIVEVK EILKSSLVNI PKDTVTLYTN	240
SGCLCPQLVA NEEYIIMGYE DKERTRLLLV EGSLAEKWRD RLAKKVKRWD QKLRRPRKSK	300
DPVAPIPNKN SNSRQARS	

Figure 3

SUBSTITUTE SHEET (RULE 26)

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GAATTCCTT TCACACAGGA CTCCTGGCAG AGGTGAATGG TTAGCCCTAT GGATTGTT	60
CTTAAGGGAA AGTGTGTCCT GAGGACCGTC TCCACTTACC AATCGGGATA CCTAAACCAA	
TGTTGATTTT GACACATGAT TGATTGCTTT CAGATAGGAT TGAAGGACTT GGATTTTAT	120
ACAACTAAAA CTGTGTACTA ACTAOCGAAA GTCTATCCTA ACTTCCTGAA CCTAAAAATA	
CTAATTCTGC ACTTTTAAAT TATCTGAGTA ATTGTTTATT TTGTATTGGA TGGGACTAAA	180
GATTAAGACG TGAAAATTTA ATAGACTCAT TAACAAGTAA AACATAACCT ACCCTGATTT	
GATAAACTTA ACTCCTTGCT TTTGACTTGC CCATAAACTA TAAGGTGGGG TGAGTTGTAG	240
CTATTTGAAT TGAGGAACGA AAAGTGAACG GGTATTTGAT ATTCCACCCC ACTCAACATC	
TTGCTTTTAC ATGTGCCCAG ATTTTCCCTG TATTCCTGT ATTCCCTCTA AAGTAAGCCT	300
AACGAAAATG TACACGGGTC TAAAAGGGAC ATAAGGGACA TAAGGGAGAT TTCAATCGGA	
ACACATACAG GTTGGGCAGA ATAACAATGT CTGGAACAAG GAAAGTGGAC TCATTACTGC	360
TGTGTATGTC CAACCCGTCT TATTGTTACA GAGCTTGTTC CTTTCACCTG AGTAATGACG	
TACTGGCCAT ACCTGGACTG GCGCTTCTCT TATTACCCAA TGCTTACTGT GCTTGGTGTG	420
ATGACCGGTA TGGACCTGAC CGCGAAGAGA ATAATGGGTT ACGAATGACA CGAAGCACAC	
AGCCTGTGCG GATCCCATG TGCAAATCTA TGCCATGGAA CATGACCAAG ATGCCCAACC	480
TOGGACACGC CTAGGGGTAC ACGTTTAGAT ACGGTACCTT GTACTGGTTC TACGGGTGCG	
ATCTCCACCA CAGCACTCAA GCGAATGCCA TCCTGGCAAT TGAACAGTTT GAAGGTTTGC	540
TAGAGGTGGT GTCGTGAGTT CGGTTACGGT AGGACCGTTA ACTTGTCAA CTTCCAAACG	
TGACCACTGA ATGTAGCCAG GACCTTTTGT TCTTTCTGTG TGCCATGTAT GCGCCCATTT	600
ACTGGTGACT TACATCGGTC CTGGAAAACA AGAAAGACAC ACGGTACATA CGGGGGTAAA	
GTACCATCGA TTTCCAGCAT GAACCAATTA AGCCTTGCAA GTCCGTGTGC GAAAGGGCCA	660
CATGGTAGCT AAAGGTGTA CTTGGTTAAT TCGGAACGTT CAGGCACACG CTTTCCCGGT	
GGGCGGCTG TGAGCCCAT CTCTAAAGT ACGGCACAC TTGGCCAGAG AGCCTGGCAT	720
CCCGGCCGAC ACTCGGTAA GAGTATTTCA TGGCCGTGTG AACCGGTCTC TOGGACCGTA	
GTGAAGAGCT GCGCGTATAT GACAGAGGAG TCTGCATCTC CCCAGAGGCT ATCGTCACAG	780
CACCTTCTGA CGGGCATATA CTGTCTCTC AGACGTAGAG GGGTCTCCGA TAGCAGTGTG	
TGGAACAAGG AACAGATTCA ATGCCAGACT TCTCATGGA TTCAAACAAT GGAAATTGCG	840
AOCTTGTTCC TTGTCTAAGT TAOGGTCTGA AGAGGTACCT AAGTTTGTTA CCTTTAAGC	
GAAGCGGCAG GGAGCACTGT AAATGCAAGC CCATGAAGGC AACCCAAAAG ACGTATCTCA	900
CTTGGCGGTC CCTCGTGACA TTTACGTTG GGTACTTCCG TTGGGTTTTT TGCATAGAGT	
AGAATAATTA CAATTATGTA ATCAGAGCAA AAGTGAAAGA GGTGAAAGTG AAATGCCACG	960
TCTTATTAAT GTTAATACAT TAGTCTCGTT TTCACTTTCT CCACTTTTAC TTTACGGTGC	
ACGCAACAGC AATTGTGGAA GTAAAGGAGA TTCTCAAGTC TTCCCTAGTG AACATTOCTA	1020
TGCGTTGTG TTAACACCTT CATTCTCTT AAGAGTTCAG AAGGGATCAC TTGTAAGGAT	

Figure 4A

SUBSTITUTE SHEET (RULE 26)

AAGACACAGT GACACTGTAC ACCAACTCAG GCTGCTTGTG CCCCCAGCTT GTTGCCAATG 1080
 TTCTGTGTCA CTGTGACATG TGGTTGAGTC CGACGAACAC GGGGGTCGAA CAACGGTTAC
 AGGAATACAT AATTATGGGC TATGAAGACA AAGAGCGTAC CAGGCTTCTA CTAGTGGAAG 1140
 TCCTTATGTA TTAATACCCG ATACTTCTGT TTCTCGCATG GTCOGAAGAT GATCACCTTC
 GATCCTTGGC CGAAAAATGG AGAGATCGTC TTGCTAAGAA AGTCAAGCGC TGGGATCAAA 1200
 CTAGGAACCG GCTTTTTTACC TCTCTAGCAG AACGATTCTT TCAGTTCGCG ACCCTAGTTT
 AGCTTCGACG TCCCAGGAAA AGCAAAGACC CCGTGGCTCC AATTCCCAAC AAAAACAGCA 1260
 TCGAAGCTGC AGGGTCCTTT TCGTTTCTGG GGCACCGAGG TTAAGGGTTG TTTTGTCTGT
 ATTCAGACA AGCGCGTAGT TAGACTAACG GAAAGGTGTA TGGAACCTCT ATGGACTTTG 1320
 TAAGGTCTGT TCGCGCATCA ATCTGATTGC CTTTCCACAT ACCTTTGAGA TACCTGAAC
 AAATAAGAT TTGCATTGTT GGAAGAGCAA AAAAGAAAT GCACCTACAGC ACGTTATATT 1380
 TTTGATTCTA AACGTAACAA CCTTCTCGTT TTTTCTTTAA CGTGATGTCTG TGCAATATAA
 CTATTGTTTA CTACAAGAAG CTGGTTTAGT TGATTGTAGT TCTCCTTTCC TTCTTTTTTT 1440
 GATAACAAAT GATGTTCTTC GACCAATCA ACTAACATCA AGAGGAAAGG AAGAAAAAAA
 TTATACTAT ATTTGCACGT GTTCCCAGGC AATTGTTTAA TTCAACTTCC AGTGACAGAG 1500
 AATATTGATA TAAACGTGCA CAAGGGTCCG TTAACAAAAT AAGTTGAAGG TCACTGTCTC
 CAGTGA CTGA ATGTCTCAGC CTAAAGAAGC TCAATTCATT TCTGATCAAC TAATGGTGAC 1560
 GTCAGTACT TACAGAGTCG GATTTCTTCG AGTTAAGTAA AGACTAGTTG ATTACCACTG
 AAGTGTGTTGA TACTTGGGGA AAGTGAAC TAAGCAATGG TAAATCAGAG AAAAGTTGAC 1620
 TTCACAACT ATGAACCCCT TTCACTTGAT TAACGTTACC ATTTAGTCTC TTTTCACTG
 CAATGTTGCT TTTCTGTAG ATGAACAAGT GAGAGATCAC ATTTAAATGA TGATCACTTT 1680
 GTTACAACGA AAAGGACATC TACTTGTTCA CTCTCTAGTG TAAATTTACT ACTAGTGAAA
 CCATTTAATA CTTTCAGCAG TTTTAGTTAG ATGACATGTA GGATGCACCT AAATCTAAT 1740
 GGTAAATTAT GAAAGTCGTC AAAATCAATC TACTGTACAT CCTACGTGGA TTTAGATTTA
 ATTTTATCAT AAATGAAGAG CTGGTTTGA CTGTATGGTC ACTGTTGGGA AGGTAAATGC 1800
 TAAAATAGTA TTTACTTCTC GACCAATCT GACATACCAG TGACAACCCCT TCCATTTACG
 CTACTTTGTC AATTCTGTTT TAAAAATTGC CTAAATAAAT ATTAAGTCCT AAATAAAAAA 1860
 GATGAACAG TTAAGACAAA ATTTTAAAG GATTATTTA TAATTCAGGA TTTATTTTTT
 AAAAAAAAAA AAAAA
 TTTTTTTTTT TTTT

Figure 4B
 SUBSTITUTE SHEET (RULE 26)

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MLLLFRAIPM LLLGLMVLQT DCEIAQYYID EEEPPGTVIA VLSQHSIFNT TDIPATNFRL	60
MKQFNNSLIG VRES DGQLSI MERIDREQIC RQSLHCNLAL DVVSFSKGFH KLLNVKVEVR	120
DINDHSPHFP SEIMHVEVSE SSSVGTRIPL EIAIDEDVGS NSIQNFQISN NSHFSIDVLT	180
RADGVKYADL VLMRELDREI QPTYIMELLA MDGGVPSLSG TAVVNIRVLD FNDNSPVFER	240
STIAVDLVED APLGYLLLEL HATDDDEGVN GEIVYGFSTL ASQEVRLFK INSRTGSVTL	300
EGQVDFETKQ TYEFEVQAQD LGPNPLTATC KTVHILDVN DNTPAITITP LTTVNAGVAY	360
IPETATKENF IALISTTDRA SGSNGQVRCT LYGHEHFKLQ QAYEDSYMIV TTSTLDRENI	420
AAYSLTVVAE DLGFFSLKTK KYITVKVSDE NDNAPVFSKP QYEASILENN APGSYITTVI	480
ARDSDSQNG KVNRYLVDK VMGQSLTTFV SLDADSGVLR AVRSLDYEKL KQLDFEIEAA	540
DNGIPQLSTR VQLNLRIVDQ NDNCPVITNP LLNNGSGEVL LPISAPQNYL VFQLKAEDSD	600
EGHNSQLFYT ILRDP SRLFA INKESGEVFL KKQLNSDHSE DLSIVVAVYD LGRPSLSTNA	660
TVKFILTDSF PSNVEVVIQ PSAEEQHQID MSIIFIAVLA GGCALLLLAI FVFACTCKK	720
AGEFKQVPEQ HGT CNEERLL STPSQSVSS SLSQSESCQL SINTESENC S VSSNQEQEQ	780
TGIKHSISVP SYHTSGWHL D NCAMSGHS HMGHISTKVQ WAKEIVTSMT VTLILVENQK	840
RRALSSQCRH KPVLTQMNQ QGSDMPITIS ATESTRVQKM GTARCNMKRA IDCLTL	

Figure 5
SUBSTITUTE SHEET (RULE 26)

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GAATTCCTCAG AGATGAACTC CTTGAGATTG TTTTAAATGA CTGCAGGTCT GGAAGGATTC	60
CTTAAGGGTC TCTACTTGAG GAACTCTAAC AAAATTTACT GACGTCCAGA CCTTCCTAAG	
ACATTGCCAC ACTGTTTCTA GGCATGAAAA AACTGCAAGT TTCAACTTTG TTTTGGGTGC	120
TGTAACGGTG TGACAAAGAT CCGTACTTTT TTGACGTTCA AAGTTGAAAC AAAAACCAAG	
AACTTTGATT CTTCAAGATG CTGCTTCTCT TCAGAGCCAT TCCAATGCTG CTGTTGGGAC	180
TTGAAACTAA GAAGTTCTAC GACGAAGAGA AGTCTCGGTA AGGTTACGAC GACAACCCTG	
TGATGGTTTT ACAAACAGAC TGTGAAATTG CCCAGTACTA CATAGATGAA GAAGAACCCC	240
ACTACCAAAA TGTTTGCTG ACACTTTAAC GGGTCATGAT GTATCTACTT CTTCTTGGGG	
CTGGCACTGT AATTGCAGTG TTGTCACAAC ACTCCATATT TAACACTACA GATATACCTG	300
GACCGTGACA TTAACGTCAC AACAGTGTTG TGAGGTATAA ATTGTGATGT CTATATGGAC	
CAACCAATTT CCGTCTAATG AAGCAATTTA ATAATTCCT TATCGGAGTC CGTGAGAGTG	360
GTTGGTTAAA GGCAGATTAC TTCGTTAAAT TATTAAGGA ATAGCCTCAG GCACCTCTAC	
ATGGGCAGCT GAGCATCATG GAGAGGATTG ACCGGGAGCA AATCTGCAGG CAGTCCCTTC	420
TACCCGTCGA CTCGTAGTAC CTCTCCTAAC TGGCCCTCGT TTAGACGTCC GTCAGGGAAG	
ACTGCAACCT GGCTTTGGAT GTGGTCAGCT TTTCCAAAGG ACACCTCAAG CTTCTGAACG	480
TGACGTTGGA CGGAAACCTA CACCAGTCGA AAAGGTTTC TGTGAAGTTC GAAGACTTGC	
TGAAAGTGA GGTGAGAGAC ATTAATGACC ATAGCCCTCA CTTTCCCAGT GAAATAATGC	540
ACTTTCAOCT CCACTCTCTG TAATTACTGG TATCGGGAGT GAAAGGGTCA CTTTATTACG	
ATGTGGAGGT GTCTGAAAGT TCCTCTGTGG GCACCAGGAT TCCTTTAGAA ATTGCAATAG	600
TACACCTOCA CAGACTTTCA AGGAGACACC CGTGGTCCTA AGGAAATCTT TAACGTTATC	
ATGAAGATGT TGGGTCCAAC TOCATCCAGA ACTTTCAGAT CTCAAATAAT AGCCACTTCA	660
TACTTCTACA ACCCAGGTTG AGGTAGGTCT TGAAAGTCTA GAGTTTATTA TCGGTGAAGT	
GCATTGATGT GCTAACCAGA GCAGATGGGG TGAAATATGC AGATTTAGTC TTAATGAGAG	720
CGTAACTACA CGATTGGTCT CGTCTACCCC ACTTTATACG TCTAAATCAG AATTACTCTC	
AACTGGACAG GGAATCCAG CCAACATACA TAATGGAGCT ACTAGCAATG GATGGGGGTG	780
TTGACCTGTC CCTTTAGGTC GGTGTATGT ATTACCTCGA TGATCGTTAC CTACCCOCAC	
TACCATCACT ATCTGGTACT GCAGTGGTTA ACATCCGAGT CCTGGACTTT AATGATAACA	840
ATGGTAGTGA TAGAOCATGA CGTCAACCAAT TGTAGGCTCA GGACCTGAAA TTACTATTGT	
GCCCAGTGTT TGAGAGAAGC ACCATTGCTG TGGACCTAGT AGAGGATGCT CCTCTGGGAT	900
CGGGTCACAA ACTCTCTTCG TGGTAACGAC AOCTGGATCA TCTCCTACGA GGAGACCOCTA	
ACCTTTTGTT GGAGTTACAT GCTACTGAAG ATGATGAAGG AGTGAATGGA GAAATTGTTT	960
TGGAAAACAA CCTCAATGTA CGATGACTGC TACTACTTCC TCACCTACCT CTTTAACAAA	
ATGGATTGAG CACTTTGGCA TCTCAAGAGG TACGTCAGCT ATTTAAAATT AACTCCAGAA	1020
TACCTAAGTC GTGAAACCGT AGAGTTCTCC ATGCAGTCGA TAAATTTTAA TTGAGGTCTT	

Figure 6A
SUBSTITUTE SHEET (RULE 26)

9/18

CTGGCAGTGT TACTCTTGAA GGCCAAGTTG ATTTTGAGAC CAAGCAGACT TAOGAATTTG GACCGTCACA ATGAGAACTT CCGGTTCAAC TAAAACTCTG GTTCGTCTGA ATGCTTAAAC	1080
AGGTACAAGC CCAAGATTTG GGCCCCAACC CACTGACTGC TACTTGTAAG GTAAGTGTTC TCCATGTTGG GGTCTTAAAC CCGGGGTTGG GTGACTGACG ATGAACATTT CATTGACAAG	1140
ATATACTTGA TGTAATGAT AATACCCCAG CCATCACTAT TACCCCTCTG ACTACTGTAA TATATGAAC ACATTACTA TTATGGGGTC GGTAGTGATA ATGGGGAGAC TGATGACATT	1200
ATGCAGGAGT TGCCTATATT CCAGAAACAG CCACAAAGGA GAACCTTTATA GCTCTGATCA TACGTCCTCA ACGGATATAA GGTCTTTGTC GGTGTTTCTT CTTGAAATAT CGAGACTAGT	1260
GCACTACTGA CAGAGCCTCT GGATCTAATG GACAAGTTCG CTGTACTCTT TATGGACATG CGTGATGACT GTCTCGGAGA CCTAGATTAC CTGTTCAAGC GACATGAGAA ATACCTGTAC	1320
AGCACTTTAA ACTACAGCAA GCTTATGAGG ACAGTTACAT GATAGTTACC AOCCTACTTT TCGTGAAATT TGATGTCGTT CGAATACTCC TGTCAATGTA CTATCAATGG TGGAGATGAA	1380
TAGACAGGGA AAACATAGCA GCGTACTCTT TGACAGTAGT TGCAGAAGAC CTTGGCTTCC ATCTGTCCCT TTTGTATCGT CGCATGAGAA ACTGTCATCA ACGTCTTCTG GAACCGAAGG	1440
CCTCATTGAA GACCAAAAAG TACTACACAG TCAAGGTTAG TGATGAGAAT GACAATGCAC GGAGTAACTT CTGGTTTTTC ATGATGTGTC AGTTCCAATC ACTACTCTTA CTGTTACGTG	1500
CTGTATTTTC TAAACCCAG TATGAAGCTT CTATTCTGGA AAATAATGCT CCAGGCTCTT GACATAAAG ATTTGGGGTC ATACTTCGAA GATAAGACCT TTTATTACGA GGTCCGAGAA	1560
ATATACTAC AGTGATAGCC AGAGACTCTG ATAGTGATCA AAATGGCAAA GTAAATTACA TATATTGATG TCACTATCGG TCTCTGAGAC TATCACTAGT TTTACCGTTT CATTTAATGT	1620
GACTTGTTGA TGCAAAAGTG ATGGGCCAGT CACTAACAC ATTTGTTTCT CTTGATGCGG CTGAACACT ACGTTTTAC TACCGGTCA GTGATTGTTG TAAACAAAGA GAACACGCC	1680
ACTCTGGAGT ATTGAGAGCT GTTAGGTCTT TAGACTATGA AAAACTTAA CAACTGGATT TGAGACCTCA TAACTCTCGA CAATCCAGAA ATCTGATACT TTTGAATTT GTTGACCTAA	1740
TTGAATTTGA AGCTGCAGAC AATGGGATCC CTCAACTCTC CACTOGGTT CAACTAAATC AACTTTAACT TCGACGCTG TTACCCTAGG GAGTTGAGAG GTGAGOGCAA GTTGATTAG	1800
TCAGAATAGT TGATCAAAAT GATAATTGCC CTGTGATAAC TAATCCTCTT CTTAATAATG AGTCTTATCA ACTAGTTTTA CTATTAAOGG GACACTATTG ATTAGGAGAA GAATTATTAC	1860
GCTOGGGTGA AGTTCTGCTT CCCATCAGCG CTCTCAAAA CTATTTAGTT TTCCAGCTCA CGAGCCACT TCAAGACGAA GGGTAGTCGC GAGGAGTTTT GATAAATCAA AAGGTCGAGT	1920
AAGCCGAGGA TTCAGATGAA GGGCACAAC CCAGCTGTT CTATACCATA CTGAGAGATC TTCCGCTCCT AAGTCTACTT CCGTGTTGA GGGTCGACAA GATATGGTAT GACTCTCTAG	1980
CAAGCAGATT GTTTGOCATT AACAAAGAAA GTGGTGAAGT GTTCCTGAAA AAACAATTAA GTTCTCTAA CAAACGGTAA TTGTTTCTTT CACCACTTCA CAAGGACTTT TTTGTTAATT	2040
ACTCTGACCA TTCAGAGGAC TTGAGCATAG TAGTTGCACT GTATGACTTG GGAAGAOCTT TGAGACTGGT AAGTCTCCTG AACTCGTATC ATCAACGTCA CATACTGAAC CCTCTGGAA	2100
CATTATOCAC CAATGCTACA GTTAAATTCA TCCTCAACGA CTCTTTTCTT TCTAACGTTG GTAATAGGTG GTTACGATGT CAATTTAAGT AGGAGTGGCT GAGAAAAGGA AGATTGCAAC	2160

Figure 6B
SUBSTITUTE SHEET (RULE 26)

10 / 18

AAGTCGTTAT	TTTGCAACCA	TCTGCAGAAG	AGCAGCACCA	GATCGATATG	TCCATTATAT	2220
TTCAGCAATA	AAACGTTGGT	AGACGTCTTC	TCGTCGTGGT	CTAGCTATAC	AGGTAATATA	
TCATTGCAGT	GCTGGCTGGT	GGTTGTGCTT	TGCTACTTTT	GGCCATCTTT	TTTGTGGCCT	2280
AGTAACGTCA	CGACCGACCA	CCAACACGAA	ACGATGAAAA	CCGGTAGAAA	AAACACCGGA	
GTACTTGTA	AAAGAAAGCT	GGTGAATTTA	AGCAGGTACC	TGAACAACAC	GGAACATGCA	2340
CATGAACATT	TTTCTTTCGA	CCACTTAAAT	TCGTCCATGG	ACTTGTTGTG	CCTTGTACGT	
ATGAAGAACG	CCTGTTAAGC	ACCCCATCTC	CCAGTCGGT	CTCTTCTTCT	TTGTCTCAGT	2400
TACTTCTTGC	GGACAATTCG	TGGGGTAGAG	GGGTCAGCCA	GAGAAGAAGA	AACAGAGTCA	
CTGAGTCATG	CCAACTCTCC	ATCAATACTG	AATCTGAGAA	TTGCAGCGTG	TCCTCTAACC	2460
GACTCAGTAC	GGTTGAGAGG	TAGTTATGAC	TTAGACTCTT	AACGTGCGAC	AGGAGATTGG	
AAGAGCAGCA	TCAGCAAACA	GGCATAAAGC	ACTCCATCTC	TGTACCATCT	TATCACACAT	2520
TTCTCGTCGT	AGTCGTTTGT	CCGTATTTCT	TGAGGTAGAG	ACATGGTAGA	ATAGTGTGTA	
CTGGTTGGCA	CCTGGACAAT	TGTGCAATGA	GCATAAGTGG	ACATTCTCAC	ATGGGGCACA	2580
GACCAACCGT	GGACCTGTTA	ACACGTTACT	CGTATTACCC	TGTAAGAGTG	TACCCCGTGT	
TTAGTACAAA	GGTACAGTGG	GCAAAGGAGA	TAGTGACTTC	AATGACAGTG	ACTCTGATAC	2640
AATCATGTTT	CCATGTCACC	CGTTTCCTCT	ATCACTGAAG	TTACTGTCAC	TGAGACTATG	
TAGTGAGAA	TCAGAAAAGA	AGAGCATTGA	GCAGCCAATG	CAGGCACAAG	CCAGTGCTCA	2700
ATCACCTCTT	AGTCTTTTCT	TCTCGTAACT	CGTCGGTTAC	GTCCGTGTTT	GGTCAOGAGT	
ATACACAGAT	GAATCAGCAG	GGTTCCGACA	TGCCGATAAC	TATTTCAGCC	ACCGAATCAA	2760
TATGTGTCTA	CTTAGTCGTC	CCAAGGCTGT	ACGGCTATTG	ATAAAGTCGG	TGGCTTAGTT	
CAAGGGTOCA	GAAAATGGGA	ACTGCACATT	GCAATATGAA	AAGGGCTATA	GACTGTCTTA	2820
GTTCCAGGTT	CTTTTACCCT	TGACGTGTAA	CGTTATACTT	TTCCCGATAT	CTGACAGAA	
CTCTGTAGCT	CCTGTATATT	ACAATACCTA	CCATGCAAGA	ATGCCTAACC	TGCACATACC	2880
GAGACATCGA	GGACATATAA	TGTTATGGAT	GGTACGTTCT	TACGGATTGG	ACGTGTATGG	
GAACCATACC	CTTAGAGACC	CTTATTACCA	TATCAATAAT	CCTGTTGCTA	ATCGGATGCA	2940
CTTGGTATGG	GAATCTCTGG	GAATAATGGT	ATAGTTATTA	GGACAACGAT	TAGCCTACGT	
GGCGGAATAT	GAAAGAGATT	TAGTCAACAG	AAGTGCAACG	TTATCTCCGC	AGAGATCGTC	3000
COGCTTATA	CTTTCTCTAA	ATCAGTTGTC	TTACGTTGTC	AATAGAGGCG	TCTCTAGCAG	
TAGCAGATAC	CAAGAATTCA	ATTACAGTCC	GCAGATATCA	AGACAGCTTC	ATCCTTCAGA	3060
ATCGTCTATG	GTTCTTAAGT	TAATGTCAGG	CGTCTATAGT	TCTGTGGAAG	TAGGAAGTCT	
AATTGCTACA	ACCTTTTAAT	CATTAGGCAT	GCAAGTGAGA	ATGCACAAAG	GCAAGTGCTT	3120
TTAACGATGT	TGGAAAATTA	GTAATCOGTA	CGTTCACTCT	TACGTGTTTC	CGTTCACGAA	
TAGCATGAAA	GCTAAATATA	TGGAGTCTCC	CCTTTCCCTC	TGATGGATGG	GGGGAGACAC	3180
ATCGTACTTT	CGATTTATAT	ACCTCAGAGG	GGAAAGGGAG	ACTACCTACC	CCCCTCTGTG	
AGGACAGTGC	ATAAATATAC	AGCTGCTTTT	TATTTGCATT	TCACTTGGGA	ATTTTTTGTT	3240
TCCTGTCAAG	TATTTATATG	TCGACGAAAG	ATAACGTAA	AGTGAACCC	TAAAAACAA	
TTTTTTACAT	ATTTATTTTT	CCTGAATTGA	ATGTGACATT	GTCCTGTCAC	CTAACTAGCA	3300
AAAAATGTA	TAAATAAAAA	GGACTTAACT	TACACTGTAA	CAGGACAGTG	GATTGATCGT	

Figure 6C
SUBSTITUTE SHEET (RULE 26)

11/18

ATTAAATCCA CAGACCTACA GTCAAATATT TGAGGGCCCC TGAAACAGCA CATCAGTCAG 3360
TAATTTAGGT GTCTGGATGT CAGTTTATAA ACTCCCGGGG ACTTTGTCGT GTAGTCAGTC

GACCTAAAGT GGCCTTTTTA CTTTTCAGC CTCCTGGGTC TGCCCTCTGT GTTAATCAGC 3420
CTGGATTTC ACGGAAAAAT GAAATCGTC GAGGACCCAG ACGGGAGACA CAATTAGTCG

CCCTGGTCAA GTCTGAGTA GGATCATGGC GTTTTATAT GCATCTCACC TACTTTGGAC 3480
GGGACCAGTT CAGGACTCAT CCTAGTACCG CAAAAATATA CGTAGAGTGG ATGAAACCTG

GTGATTTACA CATAATAGGA AACGCTTGGT TTCAGTGAAG TCTGTGTTGT ATATATTCTG 3540
CACTAAATGT GTATTATCCT TTGCGAACCA AAGTCACTTC AGACACAACA TATATAAGAC

TTATATACAC GCATTTTGTG TTTGTGTATA TATTTCAAGT CCATTTCAGAT ATGTGTATAT 3600
AATATATGTG CGTAAACAC AAACACATAT ATAAAGTTCA GGTAAGTCTA TACACATATA

AGTGCAGACC TTGTAAATTA AATATTCTGA TACTTTTTCC TCAATAAATA TTTAAAT
TCACGTCTGG AACATTTAAT TTATAAGACT ATGAAAAGG AGTTATTTAT AAATTTA

Figure 6D
SUBSTITUTE SHEET (RULE 26)

12 / 18

MVCCGPGRML LGWAGLLVLA ALCLLQVPGA QAAACEPVRI PLCKSLPWNM TKMPNHLHHS	60
TQANAILAME QFEGLLGTHC SPDLLFFLCA MYAPICTIDF QHEPIKPCKS VCERARQGCE	120
PILIKYRHSW PESLACDELP VYDRGVCISP EAIVTADGAD FPMDSSTGHC RGASSERCKC	180
KPV RATQKTY FRNNYNYVIR AKVKEVKMKC HDVTAVVEVK EILKASLVNI PRDTVNLYTT	240
SGCLCPPLTV NEEYVIMGYE DEERSRLLLV EGSIAEKWKD RLGKKVKRWD MKLRHLGLGK	300
TDASDSTQNO KSGRNSNPRP ARS.	

Figure 7
SUBSTITUTE SHEET (RULE 26)

13/18

AAGCCTGGGA CCATGGTCTG CTGCGGCCCG GGACGGATGC TGCTAGGATG GGCCGGGTTG	60
TTCGGACCCCT GGTACCAGAC GACGCCGGGC CCTGCCTACG ACGATCCTAC CCGGCCCAAC	
CTAGTCCTGG CTGCTCTCTG CCTGCTCCAG GTGCCCCGGAG CTCAGGCTGC AGCCTGTGAG	120
GATCAGGACC GACGAGAGAC GGACGAGGTC CACGGGCCTC GAGTCCGACG TCGGACACTC	
CCTGTCCGCA TCCCGCTGTG CAAGTCCCTT CCCTGGAACA TGACCAAGAT GCCCAACCAC	180
GGACAGGCGT AGGGCGACAC GTTCAGGGAA GGGACCTTGT ACTGGTTCTA CGGGTTGGTG	
CTGCACCACA GCACCCAGGC TAACGCCATC CTGGCCATGG AACAGTTCGA AGGGCTGCTG	240
GACGTGGTGT CGTGGGTCCG ATTGCGGTAG GACCGGTACC TTGTCAAGCT TCCCGACGAC	
GGCACCCACT GCAGCCCGGA TCTTCTCTTC TTCTCTGTG CAATGTACGC ACCCATTTGC	300
CCGTGGGTGA CGTCGGGCCT AGAAGAGAAG AAGGAGACAC GTTACATGCG TGGGTAAACG	
ACCATCGACT TCCAGCACGA GCCCATCAAG CCCTGCAAGT CTGTGTGTGA GCGCGCCCGA	360
TGGTAGCTGA AGGTCGTGCT CGGGTAGTTC GGGACGTTCA GACACACACT CGCGCGGGCT	
CAGGGCTGCG AGCCCATTTCT CATCAAGTAC CGCCACTCGT GGCCGGAAAG CTTGGCCTGC	420
GTCCCCACGC TCGGGTAAGA GTAGTTCATG GCGGTGAGCA CCGGCCTTTC GAACCGGACG	
GACGAGCTGC CGGTGTACGA CCGCGGCGTG TGCATCTCTC CTGAGGCCAT CGTCACCGCG	480
CTGCTCGACG GCCACATGCT GGCGCCGCAC ACGTAGAGAG GACTCCGGTA GCAGTGGCGC	
GACGGAGCGG ATTTTCCTAT GGATTCAAGT ACTGGACACT GCAGAGGGGC AAGCAGCGAA	540
CTGCCTCGCC TAAAAGGATA CCTAAGTTCA TGACCTGTGA CGTCTCCCCG TTCGTCGCTT	
CGTTGCAAAT GTAAGCCTGT CAGAGCTACA CAGAAGACCT ATTTCCGGAA CAATTACAAC	600
GCAACGTTTA CATTCCGACA GTCTCGATGT GTCTTCTGGA TAAAGGCCTT GTTAATGTTG	
TATGTCATCC GGGCTAAAGT TAAAGAGGTA AAGATGAAAT GTCATGATGT GACCGCGGTT	660
ATACAGTAGG CCCGATTTCA ATTTCTCCAT TTCTACTTTA CAGTACTACA CTGGCGGCAA	
GTGGAAGTGA AGGAAATTCT AAAGGCATCA CTGGTAAACA TTCCAAGGGA CACCGTCAAT	720
CACCTTCACT TCCTTTAAGA TTTCCGTAGT GACCATTGT AAGGTTCCCT GTGGCAGTTA	
CTTTATACCA CCTCTGGCTG CCTCTGTCCT CCACTTACTG TCAATGAGGA ATATGTCATC	780
GAAATATGGT GGAGACCGAC GGAGACAGGA GGTGAATGAC AGTTACTCCT TATACAGTAG	
ATGGGCTATG AAGACGAGGA ACGTTCCAGG TTA CTCTTGG TAGAAGGCTC TATAGCTGAG	840
TACCCGATAC TTCTGCTCCT TGCAAGGTCC AATGAGAACC ATCTTCCGAG ATATCGACTC	
AAGTGAAGG ATCGGCTTGG TAAGAAAGTC AAGCGCTGGG ATATGAAACT CCGACACCTT	900
TTACCTTCC TAGCCGAACC ATTCTTTCAG TTCGCGACCC TATACTTTGA GGCTGTGGAA	
GGACTGGGTA AAAGTATGC TAGCGATTCC ACTCAGAATC AGAAGTCTGG CAGGAACTCT	960
CCTGACCCAT TTTGACTACG ATCGCTAAGG TGAGTCTTAG TCTTCAGACC GTCCTTGAGA	

Figure 8A
SUBSTITUTE SHEET (RULE 26)

14 / 18

AATCCCCGGC CAGCACGCAG CTAAATCCTG AAATGTAAAA GGCCACACCC ACGGACTCCC	1020
TTAGGGGCCC GTCGTGCGTC GATTTAGGAC TTTACATTTT CCGGTGTGGG TGCCTGAGGG	
TTCTAAGACT GCGCTGGTG GACTAACAAA GGAAAACCGC ACAGTTGTGC TCGTGACCGA	1080
AAGATTCTGA CCGCGACCAC CTGATTGTTT CCTTTTGGCG TGTCAACACG AGCACTGGCT	
TTGTTTACCG CAGACACCGC GTGGCTACCG AAGTTACTTC CGGTCCCCTT TCTCCTGCTT	1140
AACAAATGGC GTCTGTGGCG CACCGATGGC TTCAATGAAG GCCAGGGGAA AGAGGACGAA	
CTTAATGGCG TGGGGTTAGA TCCTTTAATA TGTATATAT TCTGTTTCAT CAATCACGTG	1200
GAATTACCGC ACCCAATCT AGGAAATTAT ACAATATATA AGACAAAGTA GTTAGTGCAC	
GGGACTGTTT TTTTGCAACC AGAATAGTAA ATTAAATATG TTGATGCTAA GGTTCCTGTA	1260
CCCTGACAAG AAAACGTTGG TCTTATCATT TAATTTATAC AACTACGATT CCAAAGACAT	
CTGGACTCCC TGGGTPTAAT TTGGTGTCT GTACCCTGAT TGAGAAATGCA ATGTTTCATG	1320
GACCTGAGGG ACCCAAATTA AACCACAAGA CATGGGACTA ACTCTTACGT TACAAAGTAC	
TAAAGAGAGA ATCCTGGTCA TATCTCAAGA ACTAGATATT GCTGTAAGAC AGCCTCTGCT	1380
ATTTCTCTCT TAGGACCAGT ATAGAGTTCT TGATCTATAA CGACATTCTG TCGGAGACGA	
GCTGCGCTTA TAGTCTTGTG TTTGTATGCC TTTGTCCATT TCCCTCATGC TGTGAAAGTT	1440
CGACGCGAAT ATCAGAACAC AACATACGG AACAGGTAA AGGGAGTACG ACACTTTCAA	
ATACATGTTT ATAAAGGTAG AACGGCATTT TGAAATCAGA CACTGCACAA GCAGAGTAGC	1500
TATGTACAAA TATTTCCATC TTGCCGTAAA ACTTTAGTCT GTGACGTGTT CGTCTCATCG	
CCAACACCAG GAAGCATTTA TGAGGAAACG CCACACAGCA TGACTTATTT TCAAGATTGG	1560
GGTTGTGGTC CTTCGTAAAT ACTCCTTTGC GGTGTGTCGT ACTGAATAAA AGTTCTAACC	
CAGGCAGCAA AATAAATAGT GTTGGGAGCC AAGAAAAGAA TATTTTGCCT GGTAAAGGGG	1620
GTCCGTCGTT TTATTTATCA CAACCCCTCG TTCTTTTCTT ATAAAACGGA CCAATTCCTC	
CACACTGGAA TCAGTAGCCC TTGAGCCATT AACAGCAGTG TTCTTCTGGC AAGTTTTTGA	1680
GTGTGACCTT AGTCATCGGG AACTCGGTAA TTGTCGTCAC AAGAAGACCG TTCAAAAAC	
TTTGTTTATA AATGTATTCA CGAGCATTAG AGATGAACTT ATAACTAGAC ATCTGTTGTT	1740
AAACAAGTAT TTACATAAGT GCTCGTAATC TCTACTTGAA TATTGATCTG TAGACAACAA	
ATCTCTATAG CTCTGCTTCC TTCTAAATCA AACCCATTGT TGGATGCTCC CTCTCCATT	1800
TAGAGATATC GAGACGAAGG AAGATTTAGT TTGGGTAACA ACCTACGAGG GAGAGGTAAG	

Figure 8B
SUBSTITUTE SHEET (RULE 26)

15 / 18

ATAAATAAAT TTGGCTTGCT GTATTGGCCA GGAAAAGAAA GTATTAAAGT ATGCATGCAT 1860
TATTTATTTA AACCGAACGA CATAACCGGT CCTTTTCTTT CATAATTTCA TACGTACGTA

GTGCACCAGG GTGTTATTTA ACAGAGGTAT GTAACCTCTAT AAAAGACTAT AATTTACAGG 1920
CACGTGGTCC CACAATAAAT TGTCTCCATA CATTGAGATA TTTTCTGATA TTAAATGTCC

ACACGGAAAT GTGCACATTT GTTTACTTTT TTTCTTCCTT TTGCTTTGGG CTTGTGATTT 1980
TGTGCCTTTA CACGTGTAAA CAAATGAAAA AAAGAAGGAA AACGAAACCC GAACACTAAA

TGGTTTTTGG TGTGTTTATG TCTGTATTTT GGGGGGTGGG TAGGTTTAAG CCATTGCACA 2040
ACCAAAAACC ACACAAATAC AGACATAAAA CCCCCACCC ATCCAAATTC GGTAACGTGT

TTCAAGTTGA ACTAGATTAG AGTAGACTAG GCTCATTGGC CTAGACATTA TGATTTGAAT 2100
AAGTTCAACT TGATCTAATC TCATCTGATC CGAGTAACCG GATCTGTAAT ACTAACTTA

TTGTGTTGTT TAATGCTCCA TCAAGATGTC TAATAAAAGG AATATGGTTG TCAACAGAGA 2160
AACACAACAA ATTACGAGGT AGTTCTACAG ATTATTTTCC TTATACCAAC AGTTGTCTCT

CGACAACAAC AACAAA
GCTGTTGTTG TTGTTT

Figure 8C
SUBSTITUTE SHEET (RULE 26)

16 / 18

MVCGSPGGML LLRAGLLALA ALCLLRVPGA RAAACEPVRI PLCKSLPWNM TKMPNHLHHS	60
TQANAILAIE QFEGLLGTHC SPDLLFFLCA MYAPICTIDF QHEPIKPCKS VCERARQGCE	120
PILIKYRHSW PENLACEELP VYDRGVCISP EAIVTADGAD FPMDSNGNC RGASSERCKC	180
KPIRATQKTY FRNNYNYVIR AKVKEIKTKC HDVTAVVEVK EILKSSLVNI PRDTVNLYTS	240
SGCLCPPLNV NEEYIIMGYE DEERSRLLLV EGSIAEKWKD RLGKKVKRWD MKLRHLGLSK	300
SDSSNSDSTQ SQKSGRNSNP RQARN.	

Figure 9
SUBSTITUTE SHEET (RULE 26)

17 / 18

GGCGGAGCGG	GCCTTTTGGC	GTCCACTGCG	CGGCTGCACC	CTGCCCCATC	TGCCGGGATC	60
CCGCCTCGCC	CGGAAAACCG	CAGGTGACGC	GCCGACGTGG	GACGGGGTAG	ACGGCCCTAG	
ATGGTCTGCG	GCAGCCCCGG	AGGGATGCTG	CTGCTGCGGG	CCGGGCTGCT	TGCCCTGGCT	120
TACCAGACGC	CGTCGGGCCC	TCCCTACGAC	GACGACGCCC	GGCCCCACGA	ACGGGACCGA	
GCTCTCTGCC	TGCTCCGGGT	GCCCCGGGCT	CGGGCTGCAG	CCTGTGAGCC	CGTCCGCATC	180
CGAGAGACGG	ACGAGGCCCA	CGGGCCCCGA	GCCCCGACGC	GGACACTCGG	GCAGGCGTAG	
CCCCTGTGCA	AGTCCCTGCC	CTGGAACATG	ACTAAGATGC	CCAACCACCT	GCACCACAGC	240
GGGGACACGT	TCAGGGACGG	GACCTTGTA	TGATTCTACG	GGTTGGTGGA	CGTGGTGTCG	
ACTCAGGCCA	ACGCCATCCT	GGCCATCGAG	CAGTTCGAAG	GTCTGCTGGG	CACCCACTGC	300
TGAGTCCGGT	TGCGGTAGGA	CCGGTAGCTC	GTCAAGCTTC	CAGACGACCC	GTGGGTGACG	
AGCCCCGATC	TGCTCTTCTT	CCTCTGTGCC	ATGTACGCGC	CCATCTGCAC	CATTGACTTC	360
TCGGGGCTAG	ACGAGAAGAA	GGAGACACGG	TACATGCGCG	GGTAGACGTG	GTAAGTGAAG	
CAGCAGGAGC	CCATCAAGCC	CTGTAAGTCT	GTGTGCGAGC	GGGCCCCGCA	GGGCTGTGAG	420
GTGCTGCTCG	GGTAGTTCGG	GACATTGAGA	CACACGCTCG	CCCGGGCCGT	CCCGACACTC	
CCCATACTCA	TCAAGTACCG	CCACTCGTGG	CCGGAGAACC	TGGCCTGCGA	GGAGCTGCCA	480
GGGTATGAGT	AGTTTCATGG	GGTGAGCACC	GGCCTCTTGG	ACCGGACGCT	CCTCGACGGT	
GTGTACGACA	GGGGCGTGTG	CATCTCTCCC	GAGGCCATCG	TACTGCGGA	CGGAGCTGAT	540
CACATGCTGT	CCCCGCACAC	GTAGAGAGGG	CTCCGGTAGC	AATGACGCCT	GCCTCGACTA	
TTTCCTATGG	ATTCTAGTAA	CGGAACTGT	AGAGGGGCAA	GCAGTGAACG	CTGTAAATGT	600
AAAGGATACC	TAAGATCATT	GCCTTTGACA	TCTCCCCGTT	CGTCACTTGC	GACATTTACA	
AAGCCTATTA	GAGCTACACA	GAAGACCTAT	TTCCGGAACA	ATTACAACTA	TGTCATTTCGG	660
TTCCGATAAT	CTCGATGTGT	CTTCTGGATA	AAGGCCTTGT	TAATGTTGAT	ACAGTAAGCC	
GCTAAAGTTA	AAGAGATAAA	GACTAAGTGC	CATGATGTGA	CTGCAGTAGT	GGAGGTGAAG	720
CGATTTCAAT	TTCTCTATTT	CTGATTCACG	GTAATACACT	GACGTCATCA	CCTCCACTTC	
GAGATTCTAA	AGTCCTCTCT	GGTAAACATT	CCACGGGACA	CTGTCAACCT	CTATACCAGC	780
CTCTAAGATT	TCAGGAGAGA	CCATTGTGTA	GGTGCCCTGT	GACAGTTGGA	GATATGGTGC	
TCTGGCTGCC	TCTGCCCTCC	ACTTAATGTT	AATGAGGAAT	ATATCATCAT	GGGCTATGAA	840
AGACCGACGG	AGACGGGAGG	TGAATTACAA	TTACTCCTTA	TATAGTAGTA	CCCATACTTT	

Figure 10A
SUBSTITUTE SHEET (RULE 26)

18 / 18

GATGAGGAAC GTTCCAGATT ACTCTTGGTG GAAGGCTCTA TAGCTGAGAA GTGGAAGGAT 900
 CTACTCCTTG CAAGGTCTAA TGAGAACCAC CTTCCGAGAT ATCGACTCTT CACCTTCCTA

 CGACTCGGTA AAAAAGTTAA GCGCTGGGAT ATGAAGCTTC GTCATCTTGG ACTCAGTAAA 960
 GCTGAGCCAT TTTTCAATT CGCGACCCTA TACTTCGAAG CAGTAGAACC TGAGTCATTT

 AGTGATTCTA GCAATAGTGA TTCCACTCAG AGTCAGAAAGT CTGGCAGGAA CTCGAACCCC 1020
 TCACTAAGAT CGTTATCACT AAGGTGAGTC TCAGTCTTCA GACCGTCCTT GAGCTTGGGG

 CGGCAAGCAC GCAACTAAAT CCCGAAATAC AAAAAGTAAC ACAGTGGACT TCCTATTAAG 1080
 GCCGTTTCGT CGTTGATTTA GGGCTTTATG TTTTTCATTG TGTACCTGA AGGATAATTC

 ACTTACTTGC ATTGCTGGAC TAGCAAAGGA AAATTGCACT ATTGCACATC ATATTCTATT 1140
 TGAATGAACG TAACGACCTG ATCGTTTCCT TTTAACGTGA TAACGTGTAG TATAAGATAA

 GTTTACTATA AAAATCATGT GATAACTGAT TATTACTTCT GTTCTCTTTT TGGTTCTTGC 1200
 CAAATGATAT TTTTAGTACA CTATTGACTA ATAATGAAGA CAAAGAGAAA ACCAAAGACG

 TTCTCTCTTC TCTCAACCCC TTTGTAATGG TTTGGGGGCA GACTCTTAAG TATATTGTGA 1260
 AAGAGAGAAG AGAGTTGGGG AAACATTACC AAACCCCGT CTGAGAATTC ATATAACACT

 GTTTTCTATT TCACTAATCA TGAGAAAAAC TGTCTTTTTG CAATAATAAT AAATTAAACA 1320
 CAAAGATAA AGTGATTAGT ACTCTTTTTG ACAAGAAAAC GTTATTATTA TTTAATTTGT

 TGCTGTTACC AGAGCCTCTT TGCTGAGTCT CCAGATGTTA ATTTACTTTC TGCACCCCAA 1380
 ACGACAATGG TCTCGGAGAA ACGACTCAGA GGTCTACAAT TAAATGAAAG ACGTGGGGTT

 TTGGGAATGC AATATTGGAT GAAAAGAGAG GTTCTGGTA TTCACAGAAA GCTAGATATG 1440
 AACCCTTACG TTATAACCTA CTTTCTCTC CAAAGACCAT AAGTGTCTTT CGATCTATAC

 CCTTAAACA TACTCTGCCG ATCTAATTAC AGCCTTATTT TTGTATGCCT TTTGGGCATT 1500
 GGAATTTTGT ATGAGACGGC TAGATTAATG TCGGAATAAA AACATACGGA AAACCCGTAA

 CTCCTCATGC TTAGAAAGTT CCAATGTTT ATAAAGGTAA AATGGCAGTT TGAAGTCAAA 1560
 GAGGAGTACG AATCTTTCAA GGTTTACAAA TATTTCCATT TTACCGTCAA ACTTCAGTTT

 TGTCACATAG GCAAAGCAAT CAAGCACCAG GAAGTGTTTA TGAGGAAACA ACACCCAAGA 1620
 ACAGTGATATC CGTTTCGTTA GTTCGTGGTC CTTCAAAAT ACTCCTTTGT TGTGGGTCTT

 TGAATTATTT TTGAGACTGT CAGGAAGTAA AATAAATAGG AGCTTAAGAA AGAACATTTT 1680
 ACTTAATAAA AACTCTGACA GTCCTTCATT TTATTTATCC TCGAATTCTT TCTTGTAATAA

 GCCTGATTGA GAAGCACAAC TGAAACCAGT AGCCGCTGGG GTGTTAATGG TAGCATTCTT 1740
 CGGACTAACT CTTCTGTGTT ACTTTGGTCA TCGGCGACCC CACAATTACC ATCGTAAGAA

 CTTTGGCAA TACATTTGAT TTGTTTATGA ATATATTAAT CAGCATTAGA GAAATGAATT 1800
 GAAACCGTT ATGTAAACTA AACAACTACT TATATAATTA GTCGTAATCT CTTTACTTAA

 ATAAC TAGAC ATCTGCTGTT ATCACCATAG TTTTGTAA TTTGCTTCCT TTAAATAAAA 1860
 TATTGATCTG TAGACGACAA TAGTGGTATC AAAACAAATT AAACGAAGGA AAATTTATTT

 CCCATTGGTG AAAGTCAAAA AAAAAAAAAA AAA
 GGGTAACCAC TTTCAGTTT TTTTTTTTTT TTT

Figure 10B
 SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/10942

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : 530/300, 350; 514/2; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/300, 350; 514/2; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG (MEDLINE, BIOSIS, EMBASE, WPI, USPATFULL) AUTHOR AND WORD. search terms: e.g. cerberus, xenopus

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	BOUWMEESTER et al. Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. Nature. 15 August 1996, Vol. 382, No. 6592, pages 595-601, see entire document.	1-15

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T

later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X

document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y

document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Z

document member of the same patent family

Date of the actual completion of the international search

29 AUGUST 1997

Date of mailing of the international search report

11 SEP 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/10942

A. CLASSIFICATION OF SUBJECT MATTER: IPC (6):

A01N 37/18; A61K 38/00; C07K 1/00, 2/00, 4/00, 7/00, 14/00, 16/00, 17/00; C07H 21/02, 21/04

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